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The toxicity and kinetics of larkspur alkaloid, methyllycaconitine, in mice

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ABSTRACT: Larkspur poisoning sporadically kills from 5 to 15% of the cattle on North American mountain rangelands. Of the 40 different diterpenoid larkspur alkaloids, the one that is thought to be responsible for much of the toxicity has been identified as methyllycaconitine (MLA). Little is known of MLA toxicokinetics or excretion. The purpose of this study was to further characterize the clinical effects of MLA toxicity in mice and determine the toxicokinetics of MLA excretion. Eight groups of mice were dosed intravenously with 2.0 mg/kg of BW of MLA, killed, and necropsied at 0, 1, 2, 5, 10, 15, 30, and 60 min after injection. Treated animals were reluctant to move, trembled, and developed dyspnea, muscular twitches, and convulsions. Within several minutes, the clinical signs abated and behavior slowly returned to normal over approximately 20 min. At necropsy serum, brain, liver, kidney, and skeletal muscle were collected and frozen. Blood and tissues were extracted and analyzed for MLA with HPLC and electron spray mass spectrometry. Blood MLA elimination followed a normal biphasic redistribution and excretion pattern ($r = 0.99$) with a K of elimination of 0.0376 and half-life of 18.4 min. Other tissues had similar clearance rates. These data indicate the MLA is rapidly distributed and excreted. In mice, the clinical effects of poisoning seem to affect the central nervous system, causing dyspnea and "explosive" muscular twitches and convulsions. Because livestock commonly eat larkspur at subclinical doses, they are likely to have larkspur alkaloids in many tissues. These results suggest that animals exposed to larkspur should rapidly excrete MLA (within several hours) and that the residues in animal tissues are not likely to be a problem if animals are given several days to allow toxin clearance.

Key Words: Delphinium, Diterpenoid Alkaloids, Kinetics, Toxicity

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Introduction

The economic burden of larkspur (*Delphinium* spp.) poisoning has been estimated to be millions of dollars annually (Nielsen et al., 1994). Uncalculated losses due to increased management costs, additional fencing, and lost forage are probably much higher. The toxicity and clinical effects of larkspur poisoning have been well described, and the toxic diterpenoid alkaloids have been identified (Pfister et al., 1999). Over 40 alkaloids with differing structures and toxicity have been identified. Additionally, the amount and types of alkaloids varies greatly between the different larkspur species, within different larkspur populations, and different phenological growth stages (Manners et al., 1995). This makes predicting toxicity difficult. One of the highly toxic alkaloids, to which much of larkspur's toxicity has been attributed, is methyllycaconitine (**MLA**). Recent work has demonstrated the physiological effects of MLA as it reversibly binds and blocks the action of nicotinic acetylcholine receptors (**AchR**) (Stegelmeier et al., 1998). Cholinergic receptors from different organs and tissues, even from different individuals and species, have varying binding affinities to MLA and the other nicotinic toxins. Binding affinity has been linked to toxicity (Macallan et al., 1988; Dobelis et al., 1999).

Most range cattle ingest sublethal amounts of larkspur without showing clinical toxicosis. Though such intoxications are unnoticed, larkspur toxins may be found in animal tissues and products. The purpose of this study is to compare the clinical signs of intoxication in mice with those of cattle and to describe the simple toxicokinetics of MLA in mice after a single intravenous dose.

Materials and Methods

Animals

Forty Swiss Webster male mice (Simonson Laboratories Inc., Gilroy, CA) weighing 35 ± 4 g were randomly divided into eight groups of five. These animals were also part of the control group of a vaccination trial that will be reported separately. The mice were dosed with

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purified MLA at a rate of 2.0 mg/kg of BW in the tail vein. This dose is 70% of the LD50, and should produce clinical signs but not be lethal (Manners et al., 1995). The MLA was extracted from *Delphinium barbeyi* and verified to be >99% pure via HPLC/electron spray mass spectrometry (**ESMS**) as described in the analysis section. The clinical signs of poisoning (demeanor, movement, coordination and neuromuscular function, respiration rate, and peripheral perfusion) were closely monitored. After injection, the groups were killed at 0, 1, 2, 5, 10, 15, 30, and 60 min after injection. Immediately afterward, the mice were necropsied. Serum, brain, liver, kidney, and skeletal muscle were collected and frozen at –20°C until analyzed. All animal work was done with the approval and under the supervision of the Utah State University Animal Care and Use Committee.

Sample Extractions

Tissue Samples. Frozen tissue (brain, muscle, kidney, and liver) was allowed to thaw at room temperature. Samples (0.2 to 0.3 g) were macerated with a razor blade, transferred to tared 8-mL screw-cap vials, and an exact weight of the sample was obtained. Two milliliters of 1% H_2SO_4 was added, and each sample was homogenized for 2 min. An additional 2 mL of 1% H_2SO_4 was added to the vials and the samples were mixed for 10 min. The samples were centrifuged for 20 min at 2,800 × *g*, and then decanted into 10-mL screw-cap test tubes. Five drops of concentrated NH_4OH were then added from a Pasteur pipette to each sample. The samples were mixed and then extracted twice with 2 mL of $CHCl_3$. After each extraction, the samples were spun in a centrifuge to aid layer separation, and the chloroform layer was removed with a Pasteur pipette and filtered through anhydrous Na_2SO_4 into a clean 7-mL screw-cap vial. The solvent was removed by evaporation under a flow of nitrogen at 70°C. The samples were then stored (–20°C) until analysis by HPLC/mass spectrometry (**MS**).

Serum Samples. Frozen serum samples were allowed to thaw at room temperature and then transferred to a tared 8-mL screw cap vials. One milliliter of phosphate buffer solution (137 m*M* NaCl, 2.7 m*M* KCl, and 10 m*M* phosphate buffer pH 7.4) was then added and the samples were mixed. One drop of concentrated NH_4OH from a Pasteur pipette was then added to the samples. The samples were then extracted twice with $CHCl_3$ using a procedure identical to the one stated above for the tissue samples.

Methyllycaconitine Assay

Sample and Standard Preparation. Samples were prepared for analysis by adding 1.0 mL of methanol/20 m*M* ammonium acetate (50:50) and mixing the samples for 10 to 15 min. The samples were then filtered through 0.2-μm nylon syringe filter into HPLC autosampler vials and capped.

Two sets of MLA standards to be used for calibration were prepared from a stock solution (20 ug/mL) of MLA in $CHCl_3$ stored at –20°C. The high range set included standards at 500, 300, 150, 100, and 50 ng/mL and the low range set included standards at 50, 25, 10, and 5 ng/mL. All samples were initially analyzed with the high-range calibration by HPLC/ESMS. Samples found to contain MLA levels below 50 ng/mL were reanalyzed by HPLC/ESMS/MS with the low-range calibration set. New standards were analyzed after every 40 samples.

High-Performance Liquid Chromatography/Electrospray Mass Spectrometry Analysis. Analysis of MLA in the samples was accomplished using a HP 1100 binary HPLC system coupled to a Finnegan LCQ mass spectrometer (Finnegan MAT, San Jose, CA) with modification to the methods previously described (Turek et al., 1995; Gardner et al., 1999). Samples (20 μL) were injected with a HP 1100 autosampler onto a Betasil C18 HPLC column (100 × 2 mm, 5 μm, 100 A, Keystone Scientific, Bellefonte, PA). The column was eluted using an isocratic flow of methanol:20 m*M* ammonium acetate (65:35) at 0.5 mL/min. Retention time for MLA under these conditions was approximately 2.6 min, and the HPLC analysis was stopped after 5.5 min for a total recycle time between samples of approximately 6 min. Typically, the column was cleaned after 40 samples with 100% methanol followed by equilibration of the column with the isocratic solvent.

Flow from the HPLC was connected directly to the electrospray source of the Finnegan LCQ MS. The MS was operated in a full-scan MS mode (150 to 1,000 AMU) with the high-range calibration standards. Quantification of MLA was made from peak areas generated from the 683.3 (MH^+, for MLA) extracted ion trace. Low range samples were analyzed operating the mass spectrometer in a selected product ion MS/MS mode and peak areas from the selected product ion 651.3 ($MH^+ - 32$) trace was recorded for MLA quantitation.

Method Recovery. For the measurement of recovery, 0.3 g of the tissue analyzed was homogenized in 2 mL of 1% H_2SO_4. The sample was split into six aliquots. Methyllycaconitine standard (200 ng) was added to three of the aliquots, and the samples were analyzed as described above. The average percent recovery of the fortified samples was 95%.

Analysis and Statistics

The MLA concentrations were compared between groups and tissues using ANOVA in the GLM of SAS (SAS Inst., Inc., Cary, NC). Mean separations were determined using Duncan's multiple-range test after a significant *F*-test at $P < 0.05$ (Proc GLM).

The MLA concentrations were plotted using SigmaPlot for Windows (version 5.0, SPSS Inc., Richmond, CA) and fitted to a biexponential equation of the form:

$$C_t = Ae^{-at} + Be^{-bt}$$

The following parameters were determined:

$$Cl_t = Dose/AUC$$
$$(V_D)_\beta = Cl_T/k_{elim}$$
$$T½ = 0.693/k_{elim}$$
$$C_{max}$$
$$T_{max}$$
$$V_p = Dose/A+B$$
$$V_t = V_pXk_{12}k_{21}$$
$$k_{12} = AB(b - a)/(A+B)(Ab+Ba)$$
$$k_{21} = Ab+Ba/A+B$$

where C is the concentration of MLA in serum or tissue at time (t), A and B are preexponential coefficients, a and b are hybrid coefficients corresponding to the distribution and elimination phases, V_p and V_t are the apparent central and tissue compartment volumes, Cl_T is total-body clearance, $(V_D)_\beta$ is the apparent volume of distribution, t½ is the elimination half life, and C_{max} – T_{max} describes the concentration and time of maximal MLA concentrations. A trapezoidal method was used to determine the area under the curve (**AUC**) of a concentration vs. time graph.

Results

Within seconds of injection, the mice were reluctant to move and they sat hunched up with diffuse piloerection resulting in a scruffy appearance. Within 1 min, the mice developed muscle tremors and spastic jerky muscular convulsions. In highly susceptible animals, these jerky convulsions were followed by dyspnea, which caused the nose and toes to become cyanotic. Breathing rapidly and shallowly, the mice would lie for several minutes, after which they would slowly recover. All animals seemed to have recovered and were completely normal within 20 min. Although clinical signs were severe, none the animals included in this study developed lethal MLA toxicosis.

As seen in Table 1 and Figures 1 and 2, MLA concentrations peaked in the serum, liver, and brain within 1 min after dosing. The muscle and kidney were a little slower to reach peak concentrations, with highest MLA concentrations at 2 min. The kidney MLA concentrations were higher than other tissues at 5 min and tended to be higher through the rest of the study. The highest brain MLA concentrations were about 0.5 μg/g or about 25% the concentrations found in the other tissues.

The terminal K of elimination of MLA from the serum was 0.0397, with a half-life of 17.46 min. Similar elimination constants were found for other tissues (Table 2). The terminal elimination phase is slightly longer for the kidney and liver, with half-lives of 21.06 and 22.0 min, respectively. Kinetic parameters for the other tissues are included in Table 2.

Discussion

Methyllycaconitine produces significant neurologic changes in mice that clinically appear to be mediated by altered central nervous system dysfunction. Methyllycaconitine has a relatively high affinity to rat brain AchR receptors. It binds with lower affinity to rat muscle (Macallan et al., 1988; Stegelmeier et al., 1998; Dobelis et al., 1999). Receptor preparations from cattle brain and muscle have lower affinities (unpublished data). This binding affinity may explain the differences seen when animals are poisoned with purified MLA. Rats are very susceptible to MLA toxicity and clinical intoxication is characterized by tonic convulsions and seizures followed by respiratory paralysis (personal observations). Poisoning in cattle appears to initially affect peripheral receptors. This is seen clinically as muscular weakness. At higher doses, cattle may become

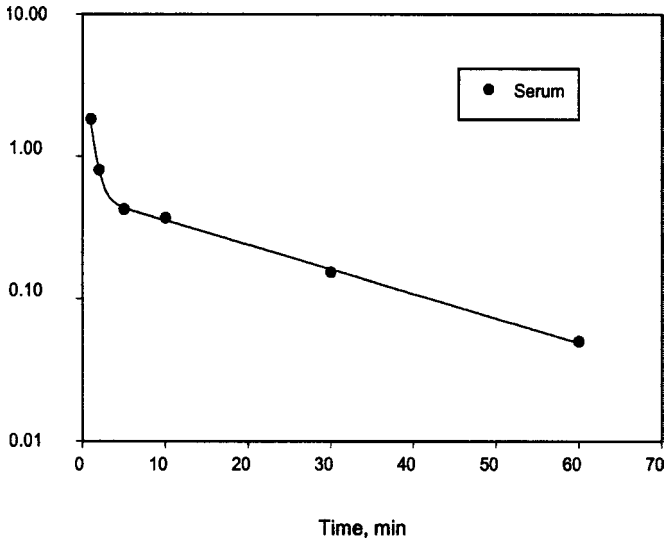

Figure 1. Serum excretion of methyllycaconitine (MLA) from mice injected intravenously with 2.0 mg/kg of BW of MLA.

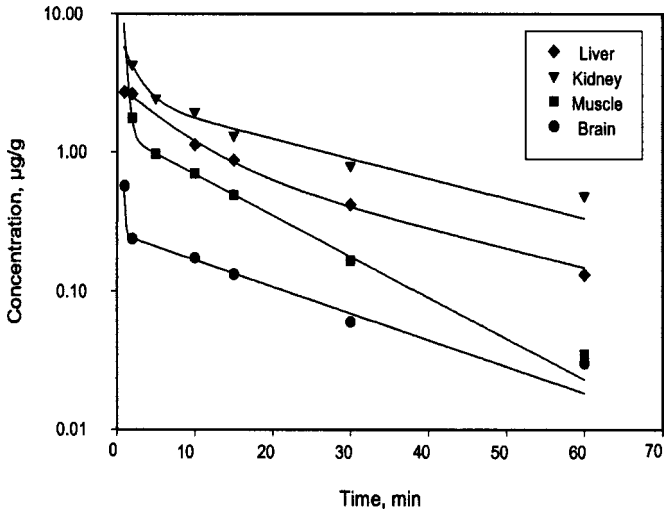

Figure 2. Excretion of methyllycaconitine (MLA) from liver, kidney, skeletal muscle, and brain of mice injected intravenously with 2.0 mg/kg of BW of MLA.

Table 1. Mean and standard deviation of methyllacconitine (MLA) concentrations in mice after intravenous injection of 2.0 mg/kg of BW of MLA

Time[a]	Serum[b]	Kidney[c]	Liver[c]	Brain[c]	Muscle[c]
0	0.01 ± 0[d]	0.02 ± 0.02[d]	0.04 ± 0.04[d]	0.01 ± 0[d]	0.00 ± 0.0[d]
1	1.82 ± 0.21[d]	2.39 ± 0.71[e]	2.72 ± 0.21[e]	0.57 ± 0.21[f]	0.59 ± 0.21[f]
2	0.80 ± 0.44[d]	4.29 ± 0.94[e]	2.64 ± 0.44[f]	0.24 ± 0.09[g]	1.76 ± 0.44[h]
5	0.43 ± 0.62[d]	2.45 ± 0.03[e]	0.80 ± 0.06[d]	0.38 ± 0.19[d]	0.97 ± 0.58[d]
10	0.37 ± 0.38[de]	1.95 ± 0.47[f]	0.99 ± 0.38[g]	0.17 ± 0.06[d]	0.70 ± 0.22[eg]
15	0.50 ± 0.19[de]	1.32 ± 0.43[f]	0.88 ± 0.19[ef]	0.13 ± 0.08[d]	0.49 ± 0.27[de]
30	0.15 ± 0.30[d]	0.64 ± 0.41[e]	0.42 ± 0.30[df]	0.06 ± 0.01[d]	0.17 ± 0.07[d]
60	0.05 ± 0.11[d]	0.49 ± 0.19[e]	0.13 ± 0.11[d]	0.03 ± 0.01[d]	0.09 ± 0.16[d]

[a]Time in minutes after intravenous MLA injection.
[b]MLA, μg/mL.
[c]MLA, μg/g.
[d,e,f,g,h]Concentrations within a time that differ between tissues have different superscript letters ($P < 0.05$).

recumbent or even fall to the ground. Some animals may struggle violently to get up. Cattle that ingest a lethal dose become laterally recumbent, bloat, and die. Death is generally attributed to paralysis of respiratory muscles; however, this may be complicated by bloat or inhalation of ingesta when animals vomit or do not properly swallow (personal observations). Receptor-binding affinity may also be responsible for species susceptibility to larkspur poisoning. Rodents and cattle are very susceptible to larkspur intoxication where as sheep are relatively resistant to poisoning (Olsen and Sisson, 1991).

MLA distribution in tissues suggests that it is quickly redistributed from the vasculature into the liver, kidney, brain, and skeletal muscle (Table 1). However, brain concentrations were only about 25% of that reached in other tissues, suggesting that MLA does not cross the blood brain barrier as easily as it crosses the vasculature of other tissues. Similar distribution studies are being conducted in other species to determine whether MLA has similarly decreased central nervous system accumulation.

The kidney has higher sustained MLA concentrations than other tissues. This suggests that MLA is actively concentrated and most likely excreted intact by the kidney. Clearance rates indicate that MLA is rapidly excreted. The half-life (t½) in the serum is about 17.46 min. Similar values have been observed in rats after a single i.v. dose (Turek et al., 1995). Renal elimination is slightly longer, with a t½ of 21.06 min and a t½ of 22.0 min in the liver. This suggests that MLA clearance of 99% from the tissue with the longest t½ (liver) would be accomplished in 154 min (seven half lives). Kinetics are likely to be vastly different after oral consumption of plant material because of the prolonged absorptive phase. Turek et al. (1995) showed that serum MLA clearances in rats dosed orally with MLA were 408 min or approximately 20 times longer than for i.v. doses. Longer clearance times are likely in animals grazing larkspur. After larkspur is in the flower and pod, cattle consume tall larkspur at relatively constant rates, which results in an equilibrium-like state. It is likely that such extended absorption phases and equilibrium states will affect elimination, which will prolong elimination of MLA. Studies in cattle are underway to determine these rates and to better define a withdrawal time in cattle under naturally occurring conditions.

Table 2. Toxicokinetic data for mice injected intravenously once with 2.0 mg/kg of BW of methyllycaconitine[a]

	Serum	Kidney	Liver	Brain	Muscle
AUC	17.11 μg/mL × min	—	—	—	—
Cl_T	116.9 mL/min × kg	—	—	—	—
V_p	334.5 mL/kg	—	—	—	—
C_t	1970.1 mL/kg	—	—	—	—
$(V_D)\beta$	2,944.8 mL/kg	—	—	—	—
K_{dist}	1,4248/min	—	—	—	—
k_{elim}	0.0397/min	0.0329/min	0.0315/min	0.0444/min	0.0682/min
t½	17.46 min	21.06 min	22.00 min	15.61 min	10.16 min
C_{max}	1.818 μg/mL	4.29 μg/g	0.57 μg/g	0.57 μg/g	1.76 μg/g
T_{max}	1 min	2 min	1 min	1 min	2 min

[a]AUC = area under the curve, Cl_t = total body clearance, V_p and V_t = the apparent central and tissue compartment volumes, $(V_D)_\beta$ = the apparent volume of distribution, K = a constant of distribution and elimination, t½ = the elimination half life. C_{max} and T_{max} describe the concentration and time of maximal MLA concentrations, respectively.

Implications

Intravenous methyllycaconitine rapidly produces clinical signs in mice that resolve in approximately 20 min. The clinical effects of poisoning seem to affect the central nervous system, causing dyspnea and explosive like muscular twitches and convulsions. Methyllycaconitine elimination followed a normal biphasic redistribution and excretion pattern with a terminal K of elimination of 0.0397 and t½ = 17.46 min for the serum. Other tissues had similar elimination rates. These data indicate the methyllycaconitine is rapidly redistributed and excreted by the mouse. Due to a prolonged oral absorptive phase, overall elimination of this toxin in livestock is likely to be longer, suggesting several days should be allowed for toxin clearance to ensure animal products are free of larkspur residues.

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